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Immunofluorescence and Herpes-Type Virus Particles in the P3HR-1 Burkitt Lymphoma Cell Line

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A subline of the P3 (Jijoye) Burkitt lymphoma cell line, designated P3HR-1, initially contained 1 to 5% of cells which were positive by indirect immunofluorescence with selected human sera. After 4 months of propagation, this cell line regularly showed 15 to 40% reactive cells. Antigen(s) in the cell line which was reactive by immunofluorescence was similar or identical to that found in several other Burkitt tumor cell lines in previous studies. When the cells were incubated at 35 or 32 C for 9 to 15 days without refeeding, more than 50% of the cells became immunofluorescence-positive. Thirteen different cultures of P3HR-1 cells, which contained up to 75% immunofluorescence-positive cells, were thin-sectioned and examined by electron microscopy. The percentage of cells containing herpes-type virus particles in the cultures varied from <3 to 78%. There was generally a good correlation between the number of immunofluorescent cells and the number of cells containing virus particles. The number of virus particles per cell section ranged from 1 to more than 100. These results strongly support the hypothesis that the immunofluorescent antigen is related to the presence of the herpes-type virus particle in the cells.

Electron-microscopic studies have revealed the presence of herpes-type virus particles in a small proportion of cells from a number of cell lines derived from Burkitt lymphomas (2, 4, 5, 15, 18). Efforts to isolate the virus from such cell lines by routine virological procedures have been generally unsuccessful in the past, although separation of the virus particles from the cells and their partial purification by physical procedures have been reported (8, 19).

Henle and Henle (7) found that indirect immunofluorescence staining with selected human sera was specific for a small proportion of the cells from several different Burkitt cell lines. The positive staining was not restricted to the sera from Burkitt tumor patients but also included many sera from healthy individuals. A higher percentage of positive sera was observed in adults than was observed in children (7, 11). It has been postulated that the immunofluorescence probably detects those cells which harbor the herpes-type virus (7, 8). One of the difficulties in investigating the nature of the immunofluorescent antigen was that the proportion of immunofluorescence-positive cells was very small, usually less than 5% in cultures of the Burkitt cell lines tested. Recently, we derived a subline of the P3 (Jijoye) Burkitt lymphoma cell line, P3HR-1, which has regularly yielded 50 to 85% immunofluorescence-positive cells when the cultures were held under certain conditions. This prompted a reinvestigation of the relationship between immunofluorescence and the virus particles in this cell line.

This paper describes the properties of the P3HR-1 subline and studies on the correlation between immunofluorescence-positive cells and the presence of virus particles in cells as revealed by electron microscopy.

MATERIALS AND METHODS

Cells. The P3HR-1 subline was used unless otherwise stated. This cell line was derived in this laboratory as a subline of Pulvertaft's P3 (Jijoye) Burkitt cells (9). The P3HR-1 cells were shown to produce the heavy chain immunoglobulin Ig M but not Ig G nor Ig A (9). The medium used was Eagle's minimal essential medium (MEM) supplemented with (per ml) 20% unheated fetal calf serum, 20 μg of L-serine, 100 μg of sodium pyruvate, 100 μg of streptomycin, and 100 units of penicillin. This medium will hereafter be referred to as M MEM. The cells were grown and maintained as stationary cultures in M MEM. Subcultures were ordinarily performed every 3 to 5 days at 37 C. Total and viable cells were counted in a hemocytometer, with trypan blue as the criterion for viability.

Preparation of cell smears for immunofluorescence staining. Cells were washed once with phosphate-

buffered saline (PBS) at *p*H 7.2 and suspended in PBS to about 5×10^5 to 10×10^5 cells/0.1 ml. A small drop of this suspension was smeared on a cover slip (1 by 4.5 cm) and allowed to dry at room temperature for approximately 30 min. The cover slips were fixed with acetone for 10 min and then dried at room temperature. The cell smears were generally used for immunofluorescence staining within 1 hr after preparation. Otherwise, they were kept in a refrigerator and used within 3 days.

Immunofluorescence staining procedure. For indirect staining, the cover slip preparations were first overlaid with the appropriate dilution of human serum and incubated for 30 min at 37 C in a humidified chamber. The cells were washed with PBS two times (5 min for each wash) and then exposed to an appropriate dilution of anti-human Ig G fluorescent antibody for 30 min at 37 C. They were again washed two times as described above and then dipped into distilled water. The excess water was drained and the cover slips were mounted on microscope slides with 50% glycerol in PBS. The preparations were examined with an American Optical microscope, with an Osram HBO 200 high-pressure mercury vapor lamp as the ultraviolet light source, a Corning 5840 or Schott BG-12 exciter filter, and a Schott GG-1 barrier filter. For direct staining, the cell smears were exposed only to fluorescein-labeled antibody. Otherwise, the procedure was the same as described for the indirect staining.

Counting of cells stained by immunofluorescence. The indirect technique with a combination of a 1:40 dilution of the VO-7 serum and 1:20 dilution of goat anti-human Ig G fluorescent antibody was used unless otherwise stated. In each specimen, the number of stained cells out of a total of approximately 1,000 cells was counted, and then the percentage of reactive cells (Fig. 1) was calculated.

Counting of cells containing virus particles by electron microscopy. Cell cultures were centrifuged at $150 \times g$ for 10 min in an International centrifuge and fixed with either 2% glutaraldehyde, followed by 1% osmium tetroxide, or Dalton's chrome-osmium fixative (1). After dehydration in graded ethyl alcohol, the cells were embedded in an Epon-Araldite mixture by the procedure of Mollenhauer (14) and thin-sectioned with glass knives on a Porter-Blum microtome. The sections were stained with 2% uranyl acetate and with lead citrate (16) and examined with a Hitachi HU-11A electron microscope. At least 20 cells in each specimen were examined, and the number of cells containing virus particles in the nucleus, in the cytoplasm, or associated with the cytoplasmic membrane was recorded. Morphological identification of virus particles was based on previous observations of others (6, 19) and from our own studies (20). Figure 2 shows an example of a cell containing virus particles.

Electron-microscopic agglutination test. The virus particles were extracted from cultures of the P3HR-1

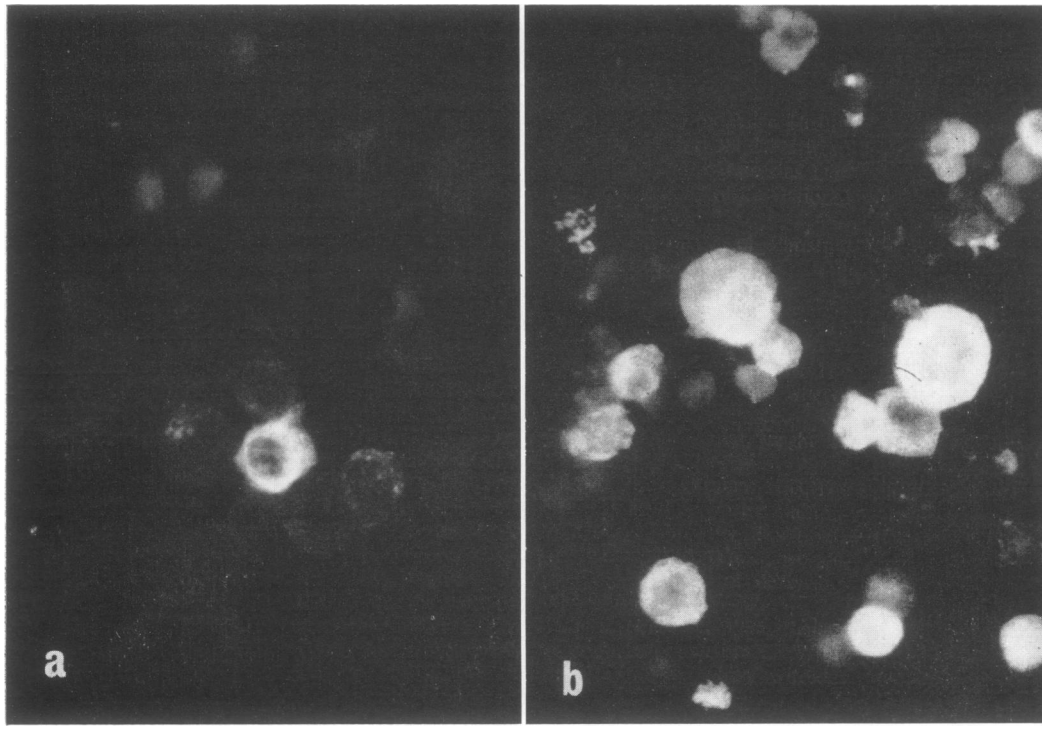

FIG. 1. *Immunofluorescence in P3HR-1 cells. (a) Cells in phase I cultured at 37 C for 3 days. (b) Cells in phase II cultured at 35 C for 12 days. Note many fluorescent but disintegrated cells.* $\times$ *310.*

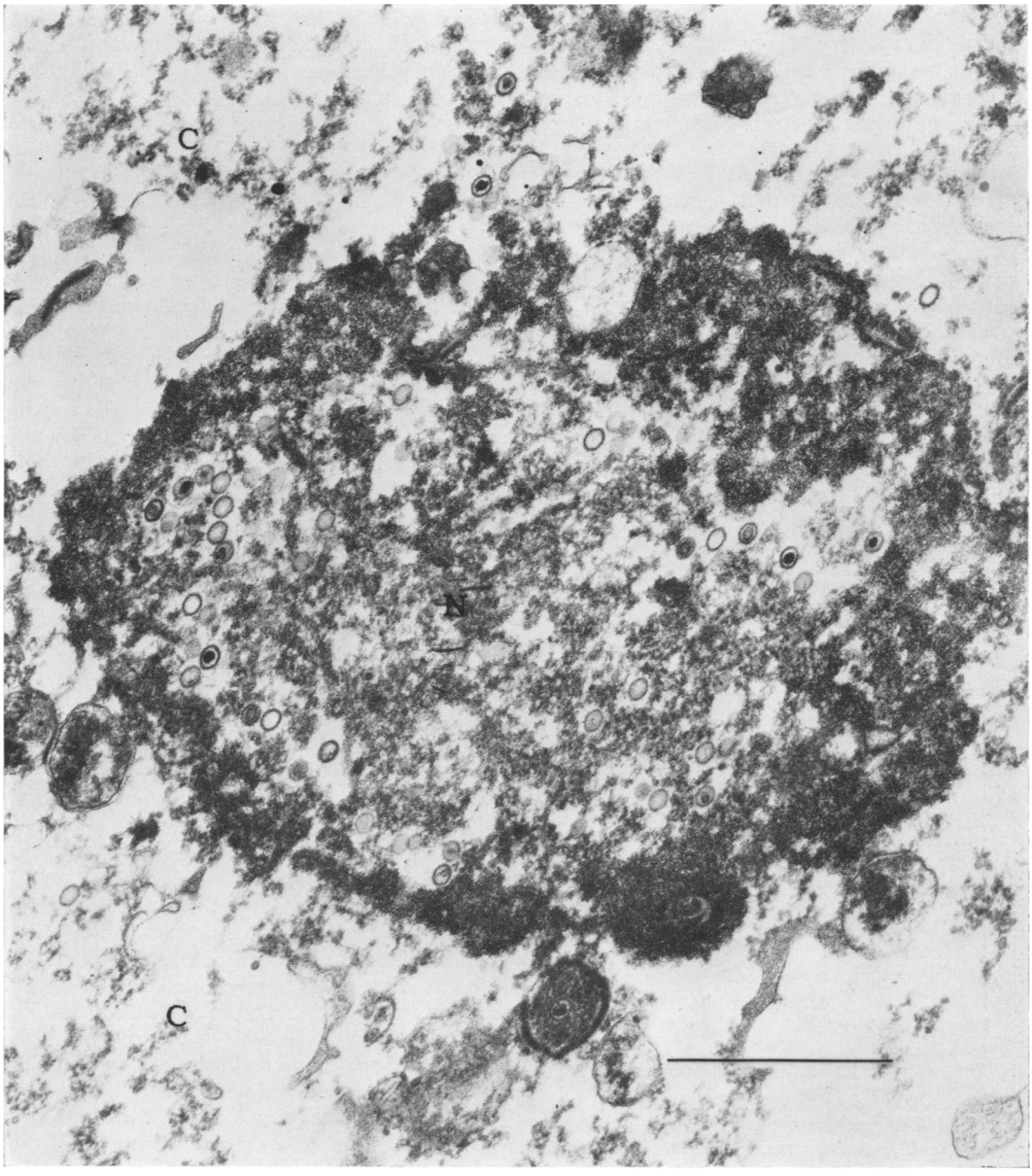

FIG. 2. *P3HR-1 cell section containing herpes-type virus particles. These cells had been incubated at 35 C for 14 days. (N) Nucleus; (C) cytoplasm. Bar represents 1.0 μ.*

cells as described previously (20). Test sera were examined by the method of Henle et al. (8).

Derivation of sublines. A semisolid agar procedure (9) was used for deriving the sublines. This procedure consisted of a modification of a soft-agar technique which has been used for cloning polyoma-transformed hamster cells (12, 17).

RESULTS

Properties of the P3HR-1 subline. During the first 4 months after derivation, the P3HR-1 subline (9) showed about 1 to 5% immunofluorescence-positive cells. After 4 months, however, the cultures began to yield a significantly higher percentage of immunofluorescence-positive cells. Serial subcultivations of the cells had been carried out regularly in M MEM every 3 to 5 days and were maintained at 37 C as stationary cultures. The changes in the percentage of immunofluorescence-positive cells in the cultures over the 6-month period following isolation of the cell line

are shown in Fig. 3. In the initial 4 months, designated as phase I, the percentage of immunofluorescence-positive cells ranged from 1 to 5%; after that time, designated as phase II, 15 to 40% of the cells were positive. This has now been maintained for over 4 months.

Cell growth in phase II was about the same as that in phase I, except that poor growth sometimes occurred during the latter period when the initial inoculum was less than 2×10^5 viable cells/ml of culture medium. There was no significant difference between the morphology of the cells in the two phases as revealed by examination with Wright-Giemsa staining and by electron microscopy. Phase II cells, however, did reveal a higher percentage of cells containing virus particles (*see* below). The percentage of Ig M-producing cells, as revealed by immunofluorescence (9), was lower in phase II cultures. The maximum in phase II was about 21%, as compared to a maximum of 50% in phase I cultures. Cells in phase II, when mixed with 10% dimethylsulfoxide and frozen in liquid nitrogen (frozen at a rate of 1 degree per min down to −40 C and stored at −170 C), were recovered as well as similarly stored cells in phase I. Cells recovered from the frozen state contained about the same proportion of immunofluorescence-positive cells as those in unfrozen cultures.

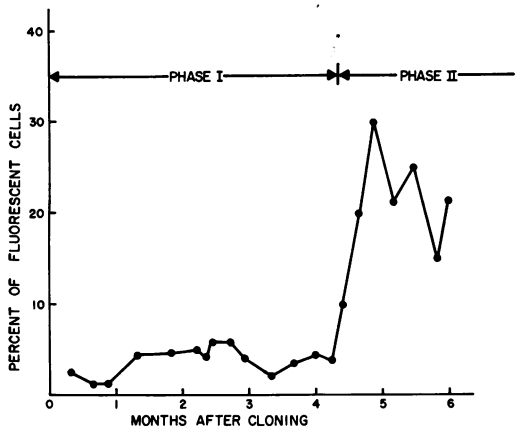

FIG. 3. *Changes in the number of immunofluorescence-positive cells in the P3HR-1 cell line during 6 months of subcultivation.*

Nature of the immunoglobulin in human sera with P3HR-1 cells. For the indirect immunofluorescence test, goat anti-human Ig G globulin conjugated with fluorescein isothiocyanate (FITC; Hyland Laboratories, Los Angeles, Calif.) was used at a 1:20 dilution in PBS. This reagent reacted with human Ig G but not with Ig M or Ig A, as revealed by immunodiffusion and immunofluorescence tests (9). Therefore, the Ig M produced by the P3HR-1 cells did not react with the fluorescent antibody. Fifty sera from healthy adults (ages ranging from 20 to 46 years) working in this Institute were tested for antibody which would react with this cell line; 48 sera gave various degrees of positive staining at the 1:5 dilution, whereas 2 did not show any specific staining at this dilution. Eleven sera showed positive staining at a 1:100 dilution. One of the positive sera with a high immunofluorescence titer was designated VO-7 serum, and this serum was used for the indirect immunofluorescence tests reported here unless otherwise stated. Three sera from Burkitt tumor patients were kindly supplied by G. Klein, Karolinska Institute, Sweden, through J. Minowada of this Institute. The titers of these three sera and of VO-7 serum, as determined by the indirect method with P3HR-1 cells, are shown in Table 1. All three Burkitt sera were positive at a dilution of 1:640, whereas the titer of the VO-7 serum was slightly lower.

To identify the nature of the reacting immunoglobulin in the four sera listed in Table 1, goat anti-human Ig G and anti-human Ig A globulins labeled with FITC were used as secondary reagents in the indirect test at 1:20 dilutions. The cells were exposed to each of the four unlabeled sera at a 1:10 dilution. Positive staining of the cells was then observed with goat anti-human Ig G fluorescent antibody but not with anti-Ig A fluorescent antibody, except for no. 13 serum which was slightly positive with anti-Ig A. This

TABLE 1. *Staining titers of the VO-7 and three Burkitt patients' sera with P3HR-1 cells in the indirect immunofluorescence test*[a]

Serum	Serum dilutions				
	1:10	1:40	1:160	1:640	1:2,560
VO-7 (normal adult)	++++	++++	++	±	−
No. 9 (Burkitt patient)	++++	++++	+++	+	−
No. 10 (Burkitt patient)	++++	++++	+++	+	−
No. 13 (Burkitt patient)	++++	++++	+++	+	−

[a] Symbols: ++++ to − indicates decreasing degrees of brilliance.

indicated that the reactive antibody in the human sera included Ig G.

The VO-7 serum globulin was coupled with FITC by the procedure of Marshall et al. (13), and was purified by chromatographic procedures (Sephadex G 75 and diethylaminoethyl cellulose columns used successively). Direct staining of the P3HR-1 cells with this antibody was observed at a 1:8 dilution. Pretreatment of the P3HR-1 cells with a 1:5 dilution of unlabeled VO-7 serum blocked the staining with labeled VO-7 globulin. Pretreatment of the cells with a 1:5 dilution of an immunofluorescence-negative serum did not block the staining by the labeled VO-7 globulin. The FITC-labeled VO-7 globulin also specifically stained the EB2 (3), EB3 (5), and B35M (Minowada et al., Cancer, *in press*) cell lines. These lines all carry a herpes-type virus. This reagent, however, did not stain the Raji (3) and 64-10 (10) cell lines, which do not have detectable virus. A human serum which had been shown by Henle to be positive for EB3 cells but negative for herpes simplex virus showed positive staining of P3HR-1 cells. Another human serum which was positive for herpes simplex virus by immunofluorescence but negative to EB3 cells gave negative results with P3HR-1 cells. Both these sera were kindly supplied by W. Henle of the Children's Hospital in Philadelphia. Absorption of the VO-7 serum with a partially purified preparation of herpes simplex virus did not remove its immunofluorescence reactivity with P3HR-1 cells. Absorption of the VO-7 serum with a crude virus preparation prepared from the P3HR-1 cells, however, removed all of the reactivity.

The VO-7 serum also agglutinated the virus extracted from the P3HR-1 cells in the electron-microscopic agglutination test described by Henle et al. (8). Absorption of the serum with partially purified herpes simplex virus did not change its ability to agglutinate the P3HR-1 virus. However, absorption of the serum with P3HR-1 virus did remove its ability to agglutinate the virus. The serum which was negative in immunofluorescence tests with P3HR-1 cells, VO-37, did not agglutinate the virus extracted from P3HR-1 cells.

A human serum containing 16 units of complement-fixing antibody against human cytomegalovirus gave completely negative immunofluorescence results with the P3HR-1 cells. The cytomegalovirus antiserum was kindly supplied by J. B. Hanshaw, University of Rochester School of Medicine. These results indicate that the immunofluorescent antigen(s) in the P3HR-1 cells is distinct from herpes simplex virus and one strain of human cytomegalovirus.

Increase in number of immunofluorescence-positive cells by prolonged cultivation. A culture of P3HR-1 cells in phase II (75% viable cells) was divided into four subcultures containing 2×10^5 viable cells/ml. The volume of each subculture was 80 ml. These cultures were incubated at 37 C (37.3 to 36.4 C), 35 C (34.2 to 35.5 C), 32 C (31.4 to 32.4 C), and 29 C (28.5 to 30.0 C), respectively. The cultures were sampled every 3 days for 3 weeks, and the total number of cells, the number of viable cells, and the percentage of immunofluorescence-positive cells were determined. The cultures were not refed during this experiment.

All of the cultures showed a near-linear increase in the percentage of immunofluorescence-positive cells with increasing time of incubation (Fig. 4). Maximal percentages were attained 9 to 18 days after initiation of the cultures. The maximal percentage of reactive cells varied with the incubation temperature. Approximately 80% were positive at 32 C, 70% at 35 C, and 60% at both 37 and 29 C. The intensity of staining was about the same in all cultures. The rate of cell growth was greater at 37 and 35 C than at 32 and 29 C during the first 3 days (Fig. 5). After 3 days, however, cell viability at 37 and 35 C was lower than at 32 or 29 C.

Correlation of positive immunofluorescence with the presence of virus particles. The availability of cell cultures with a high percentage of immuno-

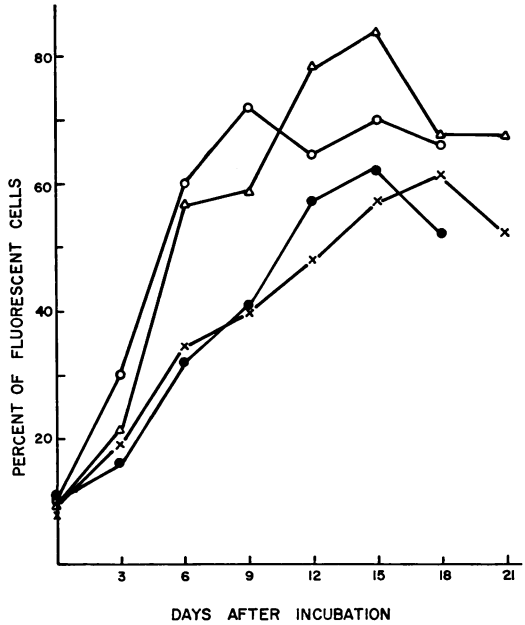

FIG. 4. *Increase in percentage of immunofluorescence-positive cells with prolonged incubation of P3HR-1 cells in phase II (see Fig. 3) at various temperatures: (●) 37 C; (○) 35 C; (△) 32 C; (×) 29 C.*

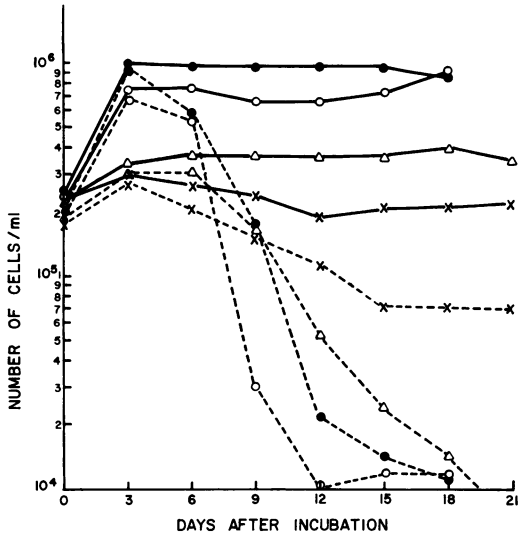

FIG. 5. *Growth and death of the P3HR-1 cells in phase II in the same experiments shown in Fig. 4.* (●) *37 C;* (○) *35 C;* (△) *32 C;* (×) *29 C; solid lines, number of total cells; broken lines, number of viable cells.*

TABLE 2. *Comparison of the number of immunofluorescence-positive cells with the number of cells containing virus particles in single cultures of P3HR-1 cells*

Cell culture	Phase of cell	Percentage of fluorescent cells[a]	Cells containing virus	
			Positive cells/no. examined	Percentage
1	I	1	0/40	<3
2	I	2	0/24	<4
3	I	3	1/33	3
4	I	5	1/25	4
5	II	30	3/28	11
6	II	37	19/68	28
7	II	40	3/23	13
8	II	45	5/51	10
9	II	51	4/36	11
10	II	67	9/29	31
11	II	73	18/23	78
12	II	74	11/33	33
13	II	75	22/32	69

[a] More than 1,000 cells were counted.

fluorescence-positive cells facilitated studies of the relationship of the number of such cells to the number of cells showing virus particles by electron microscopy. Thirteen cell cultures of P3HR-1 line in either phase I or phase II, incubated under various conditions, were examined. There was generally a good correlation between the number of immunofluorescence-positive cells and the number of cells containing virus particles (Table 2). However (except in one instance), the percentage of immunofluorescence-positive cells was always higher than the percentage of cells showing virus by electron microscopy.

DISCUSSION

The antigen(s) in the P3HR-1 line as revealed by immunofluorescence with selected human sera appears to be similar or identical to those found in the several Burkitt cell lines reported by Henle and Henle (7). A recapitulation of the observations which support this contention is as follows. (i) The P3HR-1 cells were stained by all of the sera from Burkitt lymphoma patients and by 96% of the sera from healthy adults. (ii) A serum positive for EB3 cells but negative for herpes simplex virus was positive for P3HR-1 cells. On the other hand, a serum positive for herpes simplex virus but negative to EB3 cells did not stain the P3HR-1 cells. A human serum containing antibody against cytomegalovirus did not stain the P3HR-1 cells. (iii) VO-7 serum globulin labeled with FITC, which reacts with P3HR-1 cells, also stained EB2, EB3, and B35M cell lines, which have been shown to have herpes-type particles. This same serum did not stain Raji and 64-10 cell lines, which do not contain similar virus particles. (iv) Fluorescence was seen not only in the cytoplasm but also in the nuclei. Almost all of the reactive cells showed varying stages of disintegration (7).

The correlation which was found between immunofluorescence and the presence of virus particles in the cells strongly supports the hypothesis that the immunofluorescent antigen is related to the presence of the herpes-type virus particles in the cells. The fact that an immunofluorescence-positive serum agglutinated the virus particles extracted from the cells and an immunofluorescence-negative serum did not provides additional support for this argument. It seems likely, therefore, that this type of immunofluorescence offers a convenient and reliable laboratory tool for the detection of cells containing the virus.

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